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Review

Flavonoid Evolution: An Enzymic Approach

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ABSTRACT

Flavonoid evolution in land plants is discussed from an enzymic point of view, based on the present day distribution of the major subgroups of flavonoids in bryophytes, lower and higher vascular plants. The importance of varied functions in the origin of pathways with a series of sequential steps leading to end-products is considered; it is argued that the initial function is that of an internal regulatory agent, rather than as a filter against ultraviolet irradiation. The basic synthases, hydroxylases, and reductases of flavonoid pathways are presumed to have evolved from enzymes of primary metabolism. A speculative scheme is presented of flavonoid evolution within a primitive group of algae derived from a Charophycean rather than a Chlorophycean line, as a land environment was invaded. Flavonoid evolution was preceded by that of the phenylpropanoid and malonyl-coenzyme A pathways, but evolved prior to the lignin pathway.

Flavonoid evolution in the past has been considered mainly from a chemotaxonomic or phylogenetic point of view, based on end products accumulated in various plant groups, especially within angiosperms (11). Recently, similar chemotaxonomic studies of the distribution of flavonoids within bryophytes and lower, non-seed-bearing vascular plants have been summarized (18, 19). The enzymology of the major steps leading to flavonoid subgroups has either been demonstrated in cell-free systems or has at least been characterized as to potential mechanisms. There is now a need to consider flavonoid evolution from an enzymic point of view and to speculate how such pathways leading to varied end-products may have arisen during evolution. An earlier attempt at this was made by Swain (26), but much more is now known about the enzymes involved. Enzymes of secondary metabolism have undoubtedly been derived from preexisting enzymes, ultimately from those in primary metabolism. Whereas the initial source of variation in enzyme function is due to random mutations in genes and chromosomal rearrangements, natural selection must be involved in establishing within a population a series of enzymes leading to a final functional product. A consideration of potential function(s), therefore, is all important. These functions may vary in different parts of a plant, and may have changed during evolution.

FUNCTIONS

Probably the most frequently named function of the flavonoids that arose early during the evolution of the first land plants is as a UV filter. This is an attractive concept, but because a UV filter function would require relatively large concentrations, it is difficult to argue the advantages of this function as a selective value for flavonoids during the early stages of evolution of the pathway. Presumably, the first enzymes capable of synthesizing flavonoids were not as plentiful nor as efficient as present day forms, so that large amounts of flavonoids did not accumulate initially. Also, some mechanism had to be coevolved to permit them to accumulate in the central vacuole in quantities sufficient for filtering effectiveness, or to be transported to the wall region. Ultimately, as morphological differences between different parts of a plant arose, functions might vary in root, stem, leaves, and reproductive structures.

I would like to argue that a function as internal physiological regulators or chemical messengers was the initial one, since relatively small amounts would be effective, and the site of action could be in the cytoplasm near where they were formed. Unfortunately, their action as chemical signals or agents within the intact plant is still poorly understood. However, some information is available. The most intriguing effects are the ones associated with the growth hormone, IAA. Monohydroxy B-ring flavonoids were implicated as cofactors of peroxidase functioning as an IAA oxidase that destroys the hormone, whereas dihydroxy B-ring forms inhibited the IAA degrading activity (7). More recent work has implicated both mono- and dihydroxy forms as inhibitors of IAA transport across the plasma membrane by binding to a plasma membrane protein (15). In addition, the growth inhibition in the Hepaticae (a bryophyte) by lunularic acid, a possible early stilbene or C_6-C_3 derivative, has been postulated to be comparable to that of ABA in vascular plants (8). Gottlieb (9) has also argued the primacy of internal rather than environmental factors in phytochemical evolution, but his driving force is based on a metabolic function in which less degradable secondary metabolites ultimately replenish primary metabolites; I find this a less plausible internal function.

Although phenylpropanoid phenolics could also serve as UV filters and probably were the original ones, their absorption coefficients are lower than flavonoids on a molar or weight basis. A mixture of flavanones (including their 3-hydroxy forms or dihydroflavonols), flavones and flavonols in the central vacuole of epidermal cells of leaves serves as a filter, lessening UV-B and UV-A irradiation that penetrates the earth's atmosphere with its present ozone layer. Although there is some disagreement as to the extent of the ozone layer at the presumed time of emergence of vascular plants on earth, (16) its lack or incompleteness would permit both UV-

C (<280 nm) and UV-B (280–320 nm) irradiation that could damage plants as they emerged on land. It is frequently forgotten that the protection as filters by flavonoids localized in vacuoles will be to the mesophyll cells below rather than to the epidermal cells where flavonoids are accumulated. The present day epidermal layer has been shown to absorb over 90% of UV-B radiation administered (22). While the photosynthetic machinery appears to be the key site of damage in sensitive plants, damage to growth and flowering processes has also been observed (27). PSII appears to be the most sensitive part of the photosynthetic machinery, but the actual target site is unclear (1). Whereas a mixture of flavanones, flavones, and flavonols in high enough concentration in the epidermis would provide good filter protection in the UV-B region for mesophyll cells containing chloroplasts, this cannot explain the varied glycosylation, methylation, and other substituted patterns found in present day angiosperms.

Other functions may have been utilized early in the evolution of land plants. The multiplicity of functions of flavonoids may have been crucial in the origin of such a variety of subgroups within one group of secondary compounds. In fact, the severe environmental stresses may have been a key factor in the rapid and possibly parallel evolution of flavonoid subgroups within different plant populations. In addition to acting as internal chemical signals, flavonoids could also function as chemical signals to outside organisms, permitting the evolution of mycorrhizal and symbiotic nitrogen-fixing relationships. Ultimately, constitutive flavonoids such as proanthocyanidins (25), inducible flavonoids (phytoalexins) such as pterocarpans in legumes (13), and 3-deoxyanthocyanins in sorghum (24) became effective means of chemical defense against microorganisms and, in some cases, herbivores. Finally, a function as attractants in angiosperm pollination and seed dispersal led to a vast array of the 3-hydroxy-type of anthocyanidins and their glycosides (11).

STAGES IN THE EVOLUTION OF FLAVONOID ENZYMES

Possible Evolutionary Steps in Flavonoid Enzymology

Enzymes arise from preexisting enzymes via duplication of genes and random mutations, followed ultimately by selection based on functions that may vary in time. These changes in proteins are not derived just from only single amino acid replacements. Instead, it is now believed that protein evolution also involves the piecemeal reassembly of smaller functional groups, a genes-in-pieces mechanism of protein-folding domains (12). Variations in regulatory control may occur via translocations and transposons in the promoter regions. But how did pathways with a series of sequential steps leading to an end-product evolve?

Pathways leading to organic compounds that originally existed only in the external environment are considered to have arisen in a stepwise manner in a 'backward' direction, leading to greater independence from nutrients in the external environment (14). On the other hand, pathways leading to compounds not yet found in the external environment such as secondary metabolites must have arisen in a 'forward' direction, with the requirement generally of some useful function for each intermediate that is temporarily an end-product. Such a 'forward' acquisition was postulated for the primary pathway leading to porphyrins, both respiratory and photosynthetic ones (10).

A scheme of a possible sequence of evolved enzymes leading to the aglycones of major flavonoid groups with the common 5,7-dihydroxy A-ring (Fig. 1) is given in Figure 2. The dashed lines indicate possible stages in flavonoid evolution: only levels A and B are found in some bryophytes, C is added in ferns and fern allies, and level D in tracheophytes (vascular plants). A relatively large number of flavonoid subclasses have now been identified in the bryophyte taxons, Hepaticae and Musci. However, more than 50% of bryophyte species examined do not contain detectable flavonoids. Since there are wide gaps in the distribution of flavonoid groups within taxons of the bryophytes, one has to argue either that groups such as flavonols and isoflavones and possibly even the initial chalcone synthase-flavanone steps have evolved more than once, or that numerous losses had to occur. The latter is postulated from a taxonomic point of view by Markham (19) for the Hepaticae, a bryophyte taxon. Presumably, other compounds such as terpenoids were better adapted for the functions. On the other hand, the more complex members of the isoflavonoids such as pterocarpans may have evolved only once, since a long series of sequential enzyme steps is involved. Each subgroup of aglycones can be glycosylated, methylated, acylated, prenylated, and sulfonated, but the amounts vary with each taxonomic group.

The Initial Chalcone Synthase and Isomerase Steps

The initial reaction leading to the first C_{15} compound, catalyzed by chalcone synthase, is a condensation step requiring substrates from both the phenylpropanoid and malonyl-CoA pathways. Although it may have arisen only once, more information about bryophytes as well as the enzymology of chloroflavonin biosynthesis in *Aspergillus candida* (17) is necessary. Considerable homology of angiosperm chalcone

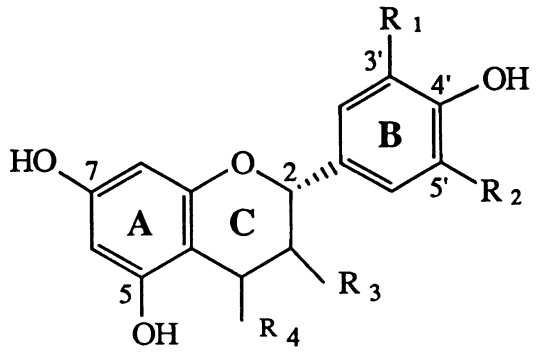

Figure 1. Basic flavonoid molecule with 5,7-hydroxylation pattern of the A-ring and 2*S* stereochemistry. With stereochemistry at C_2. Flavanone: R_3, H; R_4, C=O. 3-Hydroxyflavanone (dihydroflavonol): R_3, OH; R_4, C=O. Flavan-3-ol: R_3, OH; R_4, H. Loss of stereochemistry at C_2. Flavone: C_2=C_3; R_3, H; R_4, C=O. Flavonol: C_2=C_3; R_3, OH; R_4, C=O. Anthocyanidin: O_1=C_2 ; C_3=C_4; positive charge on C-ring; R_4, H or OH. Isoflavone: aryl migration to C_3; R_4, C=O. B-ring: R_1, R_2, H, or OH.

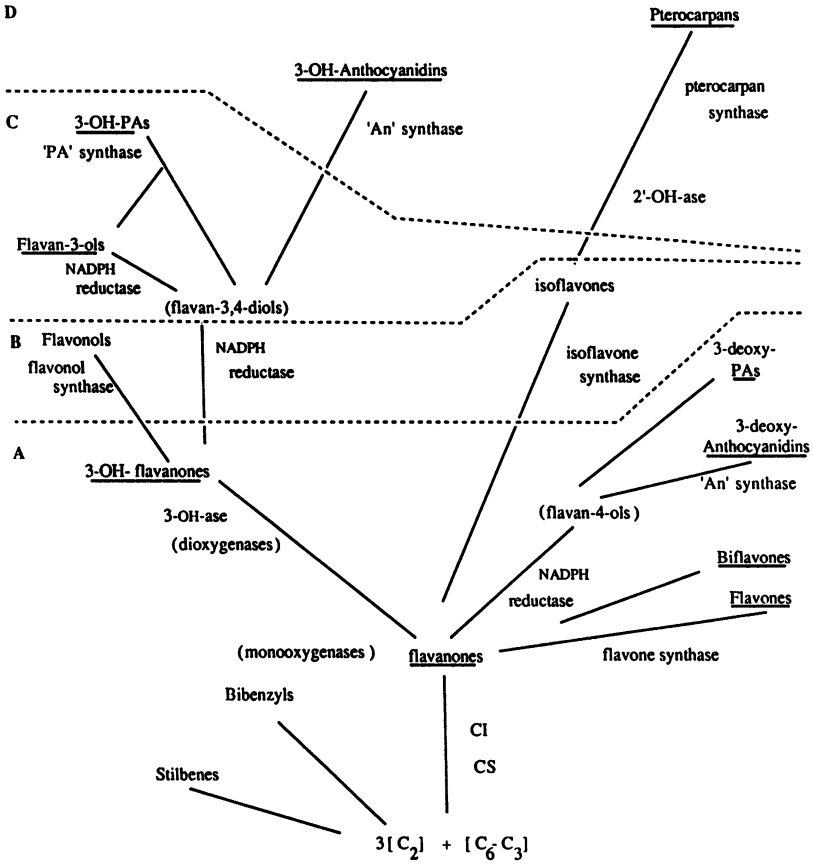

Figure 2. Evolutionary scheme of enzymic steps in the biosynthesis of the major subgroups of flavonoids with a 5,7-dihydroxy A-ring. Four levels, A, B, C, and D are shown. Levels A, B are found in bryophytes, C in ferns and fern allies, and D in gymnosperms and angiosperms. Modified from Figure 1 in Stafford ([25], p. 253). CS = chalcone synthase; CI = chalcone isomerase; PA = proanthocyanidin; An = anthocyanidin.

synthases with stilbene synthases has been found (20). It is difficult to predict which of these two enzymes might have arisen first. Stilbene synthase activity has been demonstrated in cell-free extracts in only relatively few species found in very different taxons (*Pinus*, *Arachis*, *Vitis*). In contrast to chalcone synthesis, the hydroxylation pattern of the B-ring is generally determined at the phenylpropanoid level in stilbene biosynthesis. Bibenzyl compounds, widely distributed in bryophytes, could also be formed by a stilbene synthase type of reaction (8). Because the mechanism of stepwise acquisition of C-2 units to form the A-ring may be similar to that of the β-ketoacyl-acyl carrier protein of fatty acid synthases, this enzyme might be considered the 'parent' enzyme of chalcone synthase (23). The *p*-coumaroyl-CoA can be considered to function as a primer in the subsequent condensation reactions with malonyl-CoA.

The formation of the central C-ring to form a flavanone by isomerization might originally have been nonenzymic. Since the chalcone with a 5,7-hydroxylation pattern is unstable, the first stable intermediate or product would be the flavanone. However, two flavanone isomers at C-2 are formed nonenzymically. Since present day hydroxylases of the B-ring use only one of these, the (−)-2*S* isomer (Fig. 1), the most efficient pathway would be to have enzymic control of this step so that only one stereochemical isomer is formed. The association of the chalcone synthase and isomerase in a complex would guarantee the stereochemical specificity that is now required by the next enzymes of the pathway.

Hydroxylation Patterns

Flavonoids, as well as C_6-C_3 phenylpropanoids, are characterized by three major hydroxylation patterns of the B-ring (Fig. 1). Although most chalcone synthases now use predominantly the monophenol *p*-coumarate, the first evolved chalcone synthase may have been less specific in terms of the hydroxylation pattern of the CoA-C_6-C_3 substrate, and may have used both coumaroyl and caffeoyl molecules effectively. Some chalcone synthases can still use the dihydroxy substrate, although less effectively (25). Ultimately, the advantage of limiting the substrate of the synthase to the monophenol to lessen competition between pathways with common intermediates necessitated 3′- and 5′-hydroxylation steps at the C_{15} level. These are now known as ER-localized Cyt P-450 monooxygenases. These probably arose from the ER-localized cinnamic hydroxylase of the phenylpropanoid pathway, which in turn evolved from comparable enzymes in primary metabolism.

The lack of specificity for flavanones and 3-hydroxyflavanones (dihydroflavonols) of the Cyt P-450 monooxygenase permits a grid-type pathway, or multiple paths to the same intermediate (25). However, if the enzymes are organized into an ordered sequence as an aggregate or linear multienzyme complex during biosynthesis, a single route can predominate that would be more efficient in competing for substrates. Two types of B-ring hydroxylases, both ER-localized monooxygenases, ultimately evolved: one hydroxylates only the 3′ posi-

tion, the other hydroxylates both 3′ and 5′ positions in a double step. In as much as 5′-hydroxyeriodictyol is generally not detectable, the double step hydroxylation may occur mainly at the 3-hydroxyflavanone (dihydroflavonol) rather than the flavanone level. Another explanation of the lack of accumulation of 5-hydroxyeriodictyol would be that the complex of this 3′,5′-hydroxylase with a 3-hydroxylase is extremely tight.

Subsequently, a dioxygenase type of hydroxylase, requiring 2-oxoglutarate, ascorbic acid, Fe^{2+} and O_2, was evolved, capable of hydroxylating the C-3 position of the C-ring (25). It could have been derived from the ER-localized prolyl 4-hydroxylase, an enzyme vital to the production of hydroxyproline rich proteins (4). The evolution of the above enzyme that synthesizes 3-hydroxyflavanones (dihydroflavonols) from flavanones, now permitted the synthesis of flavonols via a synthase, and both flavan-4-ols and ultimately flavan-3,4-diols (leucoanthocyanidins) via NADPH dependent reductases. The stage was now set for the synthesis of flavan-3-ols via another NADPH reductase, as well as proanthocyanidins, products now found in the ferns and fern allies. Initially, the condensation of 3,4-diols and flavan-3-ols to oligomeric proanthocyanidins could have been nonenzymic. However, a presumed condensing enzyme (proanthocyanidin synthase) has not yet been demonstrated. Only the 3-hydroxyanthocyanidins and pterocarpans are missing from these early vascular plants. The absence of the 3-hydroxyanthocyanidin pathway from the lower vascular plants (with one possible exception) is somewhat of an anomaly, since 3-deoxyanthocyanidins have been identified in a few ferns as well as mosses. The 3-deoxy-type also appears sporadically in angiosperms. Neither of the "anthocyanidin synthases" has as yet been demonstrated in cell free systems. The 3-deoxy and 3-hydroxy anthocyanidin synthases may have evolved independently; perhaps the presence of a C-3 hydroxyl group requires different enzymes.

A 2′-hydroxylase at the isoflavone level is required to initiate the most complex flavonoid group that evolved, the pterocarpans, found mainly in the leguminosae of the angiosperms (13). The aromatic ring derived from the B-ring contains generally only a mono-hydroxy group. The dihydroxy rings found in pisatin and maackiain require another hydroxlation at the 3′-position of the B-ring prior to this at the isoflavone level. Both hydroxylases are NADPH dependent microsomal monooxygenases.

NADPH Reductases

Pyridine nucleotide reductases are common to primary metabolism and presumably were modified as NADPH dependent reductases to form 4-ol and 3,4-diol precursors to proanthocyanidins and anthocyanidins. A double reductase step leads to either flavans or flavan-3-ols. Other soluble reductases produce the relatively rare 5-deoxy A-ring when attached to the chalcone synthase-flavanone complex (25), whereas another reduces 2′-hydroxydaidzein in the pterocarpan pathway (5).

Synthases—Terminal Steps Leading to Major Aglycone Subgroups

Whereas both mono- and dioxygenase types of flavone synthases have been found, only the dioxygenase type of flavonol synthase has been demonstrated so far. Hydroxylation involving hypothetical 2-OH intermediates is considered an important aspect of the mechanism postulated for these enzymes: flavone, flavonol, isoflavone, and anthocyanidin synthases. A new mechanism, however, involving a biflavanonyl biradical and a desaturase step, has been postulated for a dioxygenase type of flavone synthase (2). The mechanism for the recently purified NADPH-dependent pterocarpan synthase is still unknown, but only the more flexible isoflavanone, rather than an isoflavone, is believed capable of forming a fused furan ring (6). Neither the anthocyanidin synthase nor the proanthocyanidin condensing enzyme (proanthocyanidin synthase) has been demonstrated in cell free extracts (25).

FLAVONOID EVOLUTION IN THE FIRST LAND PLANTS—A SPECULATIVE VIEW

According to some botanists, the first land plants arose in a moist land environment from an algal Charophyceaen line, rather than directly from the aquatic Chlorophyceaen group (Fig. 3) (16). Presumably, these land invading plants developed cells with a large central vacuole for water storage such as are found in *Nitella* and in higher plants; this would also serve as the locus for the ultimate accumulation of large quantities of C_6-C_3 phenolics and the C_{15} flavonoids. Present day forms of the Charophyceae, such as *Nitella* and *Chara*, are believed to have secondarily evolved into a fresh water habitat from this primitive land group (3). The presence of flavonoids in present day algae is questionable (18).

These multicellular organisms presumably acquired hormonal control via IAA with peroxidase as an IAA oxidase to control the levels of this hormone and its transport between cells. The first modulators of this hormone that evolved might have been C_6-C_3 compounds produced by the phenylpropanoid pathway, followed by simple flavonoids such as flavanones, flavones, and then 3-hydroxyflavanones (dihydroflavonols) (25). As the capacity for the accumulation of significant quantities of these phenolics increased, and a means of accumulation within the central vacuole was devised, a function as a UV filter against UV-A and -B and as a chemical defense function could have become important subsequently. In both these functions, flavonoids were more effective than phenylpropanoids.

Within the above population(s) of pioneering land plants, bryophyte lines that synthesized mainly flavones and flavonols, branched off. A few of these also accumulated 3-deoxyanthocyanidins and isoflavones. Within other populations of early land plants, the evolution of the enzymes unique to the lignin pathway permitted the evolution of vascular plants, the tracheophytes. Proanthocyanidins and flavan-3-ols became widespread in some fern groups, while these and 3-hydroxyanthocyanidins became dominant flavonoids in gymnosperms and especially in angiosperms. Proanthocyanidins remained as major constitutive defense compounds in leaves of

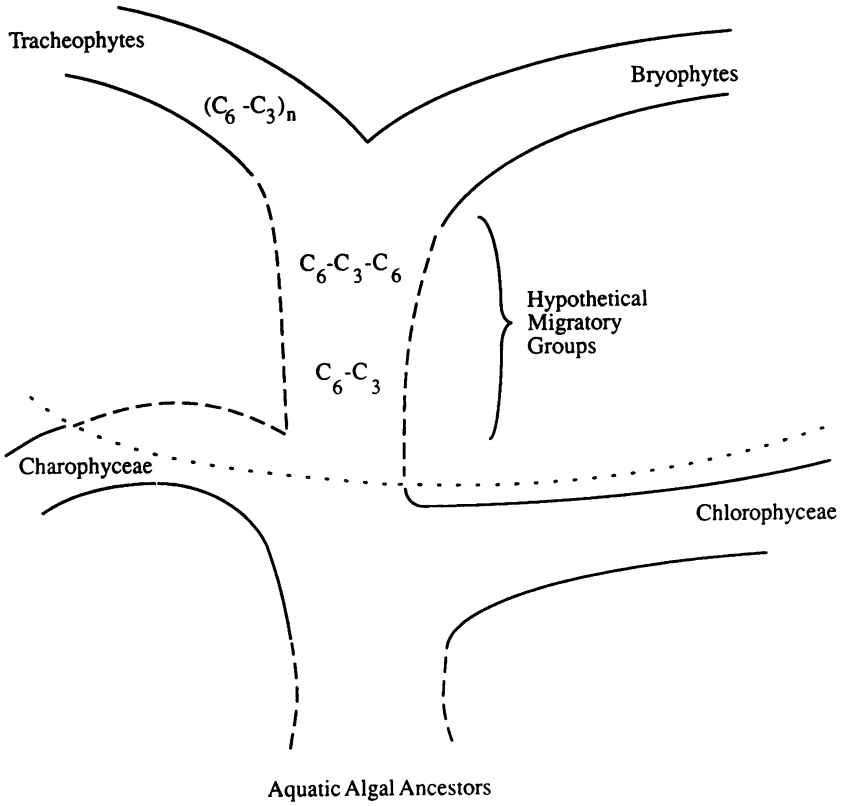

Figure 3. Divergence of Charophyceae, Chlorophyceae, bryophytes, and tracheophytes (vascular plants) from an ancestral, aquatic algal group. Dotted line: transition between aquatic and land environment. Dashed lines indicate transitional migratory groups of land plants. C_6-C_3 = phenylpropanoids; C_6-C_3-C_6 = flavonoids; $(C_6-C_3)_n$ = lignin. Modified from Chapman (3).

long-lived woody plants, but became relatively rare in short lived, herbaceous angiosperms, except in the seed coats of some of these plants. The pterocarpan pathways producing inducible phytoalexins for chemical defense purposes were evolved in a few angiosperm taxons. A diversification of flavonoid conjugates occurred within each of the major subclasses as new functions in defense and dispersal of seeds and fruits became important.

The approaches used to study the molecular biology of structural genes of the flavonoid pathway are limited so far mainly to chalcone synthases of seed plants. They need to be extended to bryophyte groups and to include stilbene and bibenzyl synthases. The homology of flavonoid enzymes to those in primary metabolism from which they might have been derived should be explored. It is crucial to understand how pathways with multiple steps, which must act in sequence in order to be effective as competitors for intermediates held in common with other pathways, are regulated.

Studies of regulatory genes in flavonoid pathways have only just started. Such genes presumably control the organization of constitutive and inducible pathways into biosynthetic units as aggregates or complexes, as well as their intertissue and intracellular distribution. A series of regulatory genes or loci controlling anthocyanin biosynthesis have been identified in maize (25). One wonders whether or not the apparent mutual exclusion within the Caryophyllales of anthocyanidins and betalains is due to a mutation in a regulatory locus, because the latter alkaloids have long wave length absorption characteristics, photoinduction requirements, and a function in pollination and seed dispersal similar to anthocyanins (21).

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